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ARTICLE

Electrochemical activation of chemotherapeutic prodrugs that mimic P450-catalyzed oxidation: proof-of-concept for a focal approach to chemical cancer treatment

Jordache Boudreau, Alicia Viloria-Petit, and Nigel J. Bunce

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Abstract: The electrochemical oxidation of substrates cyclophosphamide and acetaminophen at Ti/RuO_2 or (preferably) graphite anodes parallels P450-catalyzed oxidation in that both mechanisms involve transfer of an oxygen atom to the substrate. The aim of this work was to use this parallel to provide proof-of-concept for a localized approach to tumor chemotherapy using electrochemical oxidation to activate chemotherapeutic prodrugs ex situ. Cyclophosphamide and acetaminophen were electroactivated in batch and flow electrolytic cells and the products were tested against EMT6 mouse mammary adenocarcinoma cells. Cell viability was determined using a tetrazolium dye assay that monitored NADPH concentrations; microscopic examination showed consistent morphological differences between viable and nonviable cells. No cytotoxicity was observed in nonelectrolyzed control samples. The electrolyzed prodrugs demonstrated cytotoxicity up to the IC_{99} level at 5 mmol L^{-1} initial prodrug concentrations but not at 1 mmol L^{-1}. The long-term objective of the work is to develop an ex situ electrochemical system for activating prodrugs to cause lethal toxicity to the cells of solid tumors, many of which lack sufficient P450s to bioactivate the toxicant themselves.

Key words: electrochemical oxidation, focal chemotherapy, ex situ activation.

Résumé : L'oxydation électrochimique des substrats cyclophosphamide et acétaminophène au niveau d'anodes de Ti/RuO_2 ou (de préférence) de graphite est analogue à l'oxydation catalysée par le cytochrome P450, en ce sens que les deux mécanismes comprennent le transfert d'un atome d'oxygène au substrat. L'étude avait pour but d'exploiter ce parallèle en vue de fournir une validation de principe d'une approche localisée de la chimiothérapie des tumeurs en faisant appel à l'oxydation électrochimique pour activer ex situ les promédicaments chimiothérapeutiques. Le cyclophosphamide et l'acétaminophène ont été électroactivés dans des cellules électrolytiques en discontinu et en continu, et les produits ont été testés sur des cellules d'adénocarcinome mammaire EMT6 de souris. On a déterminé la viabilité des cellules au moyen d'un dosage colorimétrique au tétrazolium pour suivre les concentrations de NADPH; l'examen microscopique a révélé des différences morphologiques cohérentes entre les cellules viables et non viables. Aucune cytotoxicité n'a été observée dans les échantillons témoins non électrolysés. Les promédicaments électrolysés présentaient une cytotoxicité allant jusqu'au niveau IC_{99} à une concentration initiale de promédicament de 5 mmol L^{-1} mais non de 1 mmol L^{-1}. L'objectif de long terme des travaux est de développer un système électrochimique ex situ permettant d'activer des promédicaments en vue de produire une toxicité létale envers les cellules de tumeurs solides qui, souvent, ne contiennent pas suffisamment de cytochromes P450 pour bioactiver elles-mêmes les agents toxiques. [Traduit par la Rédaction]

Mots-clés : oxydation électrochimique, chimiothérapie localisée, activation ex situ.

Introduction

Breast, prostate, colon, and lung cancers present as solid tumors. For surgically accessible tumors, resection is the primary treatment option, with highest recovery rates for patients diagnosed with pre- or locally metastatic disease.[1] However, to safeguard against latent cancerous cells, most cancer patients still require chemotherapy or radiation therapy, both of which cause substantial systemic toxicity to the patient. Radiation damages healthy tissue en route to the tumor, even though different beam trajectories are used to minimize this effect, and causes the release of cytokines and hydrolytic enzymes, which exacerbate endemic toxicity.[2] Chemotherapeutic agents exploit the tendency of cancer cells to divide more rapidly than those of most normal tissues, but non-target cells are also affected, as witnessed by the hair loss and gastrointestinal disturbances that typically accompany chemotherapy, often causing the patient great suffering.

Localized chemotherapy would allow significantly higher concentrations of a toxic agent to be delivered to a tumor that was itself localized while minimizing the whole-body dose. For example, the standard dose of cyclophosphamide is 15 mg/kg body weight, or 1.05 g administered intravenously to a 70 kg patient.[3] Assuming a simplistic one-compartment distribution model, only 0.75 mg (0.07%) of cyclophosphamide would be delivered to a hypothetical 50 g tumor. A localized treatment would offer the advantage that a much higher local concentration could be achieved for a much smaller total dose, allowing greater toxicity to the tumor cells while minimizing systemic side effects. For example, isolated limb perfusion and infusion, in which a tourniquet is applied to a cancer-containing limb during infusion of chemotherapy into the limb's circulatory system, have achieved significant success in treating solitary tumors and local metastases by delivering high doses of drugs in the extremities while

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mitigating systemic damage.[4] Localized treatment would also remove the need to focus on drugs that interfere with cell division; in principle, any cytotoxic agent could be used locally to eliminate the diseased cells.

The present work explores the concept of electrochemically activated chemotherapy (EAC) in the context of intratumor injection using mouse mammary adenocarcinoma cells as a model system. EAC differs conceptually from electrochemotherapy, which utilizes a high potential gradient to disrupt cell membranes between implanted electrodes to increase the efficiency of drug uptake into target cells.[5–7] To avoid both the complexity of implanting electrodes into a patient and the likely fouling of an anode by oxidation of cellular constituents, EAC is envisaged to involve the electrochemical conversion of a prodrug ex situ to the patient.

Implementation of EAC would depend on the use of the focal approaches already used in the oncology clinic. In prostate cancer brachytherapy, radioactive pellets are implanted within the prostate gland.[8] Photothermal laser ablation therapy has been used successfully to treat early-stage and low-volume prostate tumors by heating them to the point of cytotoxicity with an infrared laser beam[9,10] or with ultrasound.[11] In photodynamic therapy, infrared radiation is directed into a tumor using a fibre optic probe. This causes electronic excitation of a systemically administered porphyrin, resulting in the conversion of ground-state $^3O_2$ in the tissue to $^1O_2$, which causes localized cellular damage.[12,13] A biological approach that parallels EAC used selective bioactivation of the cyclophosphamide analogue ifosfamide by genetically modifying cells to express high levels of CYP3A4, the P450 isoform responsible for ifosfamide bioactivation.[14–16] These modified cells, implanted into inoperable pancreatic tumors, gave significant reductions of tumor volume and overall mortality after systemic treatment with ifosfamide compared with systemic ifosfamide administration alone.

To date, there is no approach to focal cancer treatment based on direct electrochemical modification of a prodrug, although there has been in-depth discussion of the concept.[17] Our objective was to use electrochemical oxidation to mimic bioactivation of the prodrugs by cytochrome P450s, since many chemotherapeutic agents are oxidatively converted to their bioactive form by P450 monoxygenases. In this paper, we present observations on the electrochemical activation of cyclophosphamide and acetaminophen and the cytotoxicity of their oxidation products towards a mouse mammary cancer cell line in vitro. These prodrugs were selected because their biochemical mechanisms of action are already well established in the literature. Cyclophosphamide interferes with cell replication through DNA alkylation,[18] while acetaminophen causes nonspecific oxidative damage.[19,20]

Materials and methods

All chemicals listed were purchased at >99% purity. *N*-(4-hydroxyphenyl)ethanamide (acetaminophen (AP)), cyclohexa-2,5-diene-1,4-dione (*p*-benzoquinone (BQ)), (RS)-*N*,*N*-bis(2-chloroethyl)-1,3,2-oxazaphosphinan-2-amine-2-oxide (cyclophosphamide (CP)), sodium (diethylcarbamothioyl)sulfanide (diethyldithiocarbamate (DDTC)), silver(I) oxide, sodium sulfate, sodium perchlorate, ammonium ethanoate (ammonium acetate), (3*Z*,3′*Z*)-3,3′-[(3,3′-dimethylbiphenyl-4,4′-diyl)di(1*Z*)hydrazine-2-yl-1-ylidene]bis(5-amino-4-oxo-3,4-dihydronaphthalene-2,7-disulfonic acid) (trypan blue dye), and grade 60 silica gel were supplied by Sigma-Aldrich (St. Louis, Missouri). Monobasic sodium phosphate, sodium hydroxide, ethanol, HPLC-grade methanol, acetone, dichloromethane (methylene dichloride), hydrochloric acid, monosodium citrate, ethoxyethane (ethyl ether), chloroform, trypsin (1×, 0.25%), Dulbecco's modified Eagle's medium, 2-amino-2-hydroxymethyl-propane-1,3-diol, fetal bovine serum, and alumina-silicate molecular sieves (4 Å) were supplied by Fisher Scientific (Fair Lawn, New Jersey). Penicillin–streptomycin (100×) and phosphate-buffered saline solutions (1×, pH 7.4) were obtained from Invitrogen (Grand Island, New York). CellTiter 96AQ$_{ueous}$ One Solution Cell Proliferation Assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay) was obtained from Promega (Madison, Wisconsin). HPLC-grade acetonitrile was purchased from Caledon Laboratories (Georgetown, Ontario). Sodium hypochlorite (6%) was purchased from No-Frills (Guelph, Ontario), a subsidiary of Loblaws Inc., (Toronto, Ontario). Activated carbon was obtained from Calgon Carbon Corporation (Pittsburgh, Pennsylvania). All solutions were prepared using a Millipore Milli-Q Reagent Water System with water having resistivity of approximately 18.2 MΩ cm. EMT6 cells were a gift from Dr. Robert S. Kerbel (Sunnybrook Health Sciences Centre, Toronto, Ontario).

Cell culture

Sterile 100 mm × 15 mm polystyrene cell culture dishes were obtained from Fisher Scientific (Fair Lawn, New Jersey) and 96-well polystyrene tissue culture plates and 15 mL centrifuge tubes from Celltreat Scientific Products (Shirley, Massachusetts). Cytotoxicity experiments were analyzed using a Bio-Tek μQuant 96-well plate analyzer. All cell culture solutions were stored at 4 °C and warmed to room temperature before use. EMT6 cell cultures were maintained in a supplemented growth medium containing 10% (*v*/*v*) fetal bovine serum, 1% (*v*/*v*) penicillin–streptomycin (100×), and 89% (*v*/*v*) Dulbecco's modified Eagle's medium. The cells were maintained at 37 °C and 5% atmospheric CO_2 in a humidified incubator.

Confluent cells were trypsinized and suspended in 3.0 mL of supplemented growth medium, which was then centrifuged at 350 rcf for 5 min at room temperature. The pellet was resuspended in 10.0 mL of supplemented assay medium containing 1% (*v*/*v*) fetal bovine serum instead of 10%. A 1.0 mL aliquot was plated into a new culture dish containing supplemented growth medium to maintain a colony.

From the 10.0 mL of supplemented assay medium suspension, an aliquot diluted in trypan blue (1:1) was used to count cells using a Neubauer hemocytometer. Only cell suspensions with 95% or higher cell viability, as shown by trypan blue exclusion, were used for subsequent assay. An aliquot of the 10.0 mL of supplemented assay medium suspension was then taken and diluted to 10.0 mL with fresh supplemented assay medium so that a 100.0 μL aliquot of the diluted suspension would contain 5000 cells. This diluted suspension was then plated into a 96-well plate and the cells were left to incubate overnight. Cells were inspected microscopically for uniform distribution.

HPLC analysis

Electrolyses were followed by HPLC using a Waters 600E pump system equipped with a Waters 2487 dual λ absorbance detector with a silica precolumn guard (Waters Corp., Milford, Massachusetts). Samples were manually injected into the 20 μL sample loop of a Rheodyne injector. AP experiments used a reverse-phase Inertsil Sulfa C_{18} column (150 mm × 4.6 mm, 5 μm particle size) (GL Sciences, Inc., Torrance, California). Experiments with cyclophosphamide employed a Waters reverse-phase C_{18} μBondapack column (300 mm × 3.9 mm, 10 μm particle size). Chromatographic analysis was performed with Millenium-32 Chromatography Manager software from Waters. For AP, an isocratic mobile phase of 30:70 acetonitrile–water was used at a flow rate of 1.0 mL min^{-1}; absorbance was read at 254 nm. For CP, the gradient-elution method was modified from Cummings et al.[21] Solvent A was 100% acetonitrile and solvent B was milliQ water; the gradient was 30:70 A–B, time 0–5 min, linear increase to 100% solvent A by 10 min, and restoration of the original 30:70 mixture during 10–15 min. The flow rate was 1.0 mL min^{-1} and absorbance was read at 275 nm.

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Batch cell electrolysis

The cathode was a 1.0 mm diameter Ti wire; anodes were Ti/RuO_2 (1.5 × 5.0 cm) or graphite rods (d = 1.5 cm) purchased from De Nora Tech. (Painsville, Ohio) and Fisher (St. Louis, Missouri), respectively. Solutions were stirred during electrolysis using a Thermix stirrer (Fisher Scientific model 120 MR). Power to the electrochemical reactor was supplied by an EG&G model 363 potentiostat/galvanostat. Voltammetric data were obtained using an EG&G model 273A potentiostat/galvanostat controlled using Power Suite software (version 2.12.1) at a fixed sweep rate of 50 mV s^{-1}.

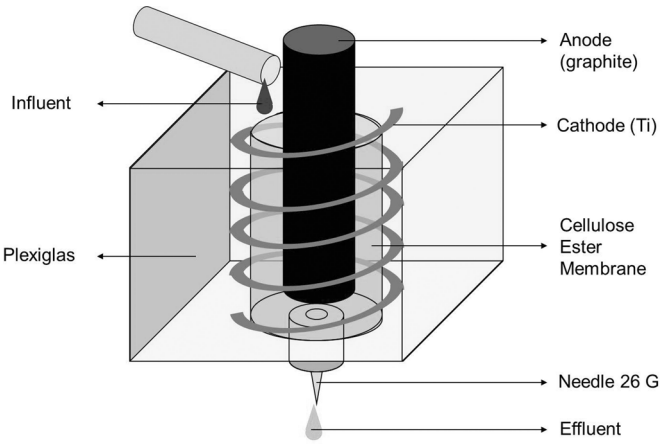
Batch cell electrolyses were carried out in 100 mL Pyrex beakers with a solution volume of 50 mL. The cell was divided, to prevent back reduction of oxidized products, with a porous ceramic frit fitted inside a glass tube, which contained the titanium wire cathode. The anodes had submerged areas of 3.7 cm^2 (Ti/RuO_2) and 6.1 cm^2 (graphite). Solutions were prepared afresh before electrolysis using milliQ water, 25 mmol L^{-1} sodium sulfate, and 1.0 or 5.0 mmol L^{-1} prodrug. The catholyte was also 25 mmol L^{-1} sodium sulfate. Potentiostatic electrolyses used a saturated calomel reference electrode (SCE) (Fisher Scientific, Toronto, Ontario) in the anodic compartment of the cell. After each experiment, anodes were visually inspected for polymer deposition and cleaned with either ethanol or acetone.

CP experiments were carried out for 90 min with 1 mL samples collected every 30 min. These were immediately treated with 100 μL of 1.0 mg mL^{-1} DDTC in glass vials, lightly agitated by vortex, placed in a 70 °C water bath for 30 min, and then cooled to room temperature for HPLC analysis. CP, phosphoramide mustard (PM), and DDTC are all transparent at 490 nm, the wavelength used to analyze the PM–DDTC adducts. Time zero samples were not electrolyzed. Samples intended for toxicological analysis were not derivatized. Materials or solutions that came into contact with CP, a known carcinogen, were exposed to 6% hypochlorite to destroy the compound prior to disposal.[22] Crystalline and aqueous solutions of CP were weighed and handled in a fume hood using manufacturer's precautions.

Flow cell

The design (Fig. 1) was modified from Miner and Kissinger.[23] A Plexiglas cell (3.0 × 3.6 × 4.7 cm, total capacity 51 cm^3) was constructed by gluing each panel with methylene chloride. The Vycor glass separator between the anode and cathode compartments in the original design was replaced by a 1.0 mL capacity, 0.5 kDa Spectra/Por Float-A-Lyzer G2 dialysis device, which served as the anode compartment and was similarly glued inside the cell. A 2.0 mm diameter hole was drilled through both the Float-A-Lyzer and Plexiglas cell to provide membrane rigidity and to serve as an attachment point to the cell. A 1 cm portion of a 10.0 mL syringe tip was attached to the bottom of the cell where a 2.0 mm hole was drilled to allow effluent to escape from the anode chamber, with the flow controlled by a 26 gauge Luer-lok needle. The cathode was a titanium wire wrapped helically around the Float-A-Lyzer. A graphite rod (diameter 4.0 mm) was the preferred anode. The cathodic compartment was filled before each experiment with 50 mL of fresh 25 mmol L^{-1} sodium sulfate. A peristaltic pump was used to provide influent of AP or CP solutions to the anode compartment. Flow through the anode compartment was gravity driven and restriction controlled with a portion of a Luer-lok syringe fastened to the bottom of the Plexiglas cell, in-line with the previously drilled hole. This was convenient for exchanging the 26 gauge needle tips, which were chosen to produce uniformly sized drops of effluent (5.5 ± 0.2 mg). Flow rate was measured by determining the time needed to collect 5.0 mL of effluent in a volumetric flask. Effluent samples were analyzed by HPLC to determine when a steady state had been achieved.

Fig. 1. Schematic depiction of the constructed flow cell used in experimentation; separation of the anode and cathode compartments prevented desired oxidation products from redox cycling. Dimensions: 3.0 cm × 3.6 cm × 4.7 cm, cathode chamber volume 51 mL, anode chamber volume 1.0 mL.



Toxicity assays

The toxicity of a solution was assessed by introducing 50.0 μL of solution to a well containing 5000 cells per 100 μL and incubating for 24 h; milliQ water (50.0 μL) served as the control treatment in all toxicity experiments. MTS reagent (20.0 μL) was added to each well of the 96-well plate and incubated for 2 h; absorbance was read at 490 nm using the 96-well plate reader. All experiments and controls were performed in triplicate.

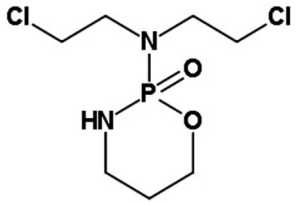
To test the electrolyzed solutions, 2.0 mL of electrolyzate was collected after 15 and 30 min of electrolysis of both CP and AP using both 1 and 5 mmol L^{-1} starting concentrations (graphite anode, 20 mA). Subconfluent cells (plated the day before at a density of 5000 cells per 100 μL of growth medium) were then treated with 50 μL of electrolyzate (150 μL total volume). After 24 h incubation at 37 °C and 5% CO_2, 20 μL of MTS reagent was added to the cells. After incubation under the same conditions for an additional 2 h, absorbance was read at 490 nm. Treatments and assays were performed in triplicate.

The toxicity data for BQ were treated by the unweighted probit method to obtain the IC_{50} and IC_{99} values. These assays were analyzed statistically using a Student *t* test by assessing homogeneity of the data as an average of nontoxic results (across experiments) versus the individual toxic response. For $T_{value} > T_{0.95}$, the toxic response was statistically significant at 95% confidence. Toxicity data were expressed as a percentage of viability relative to naive controls (50.0 μL of milliQ water). In the cytotoxicity experiments with electrolyzed prodrugs, 100 μL of growth medium was treated with 50 μL of electrolyzate, and so the concentrations to which the EMT6 cells were exposed were one thirdof those analyzed by HPLC.

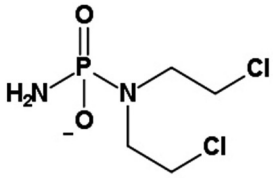
Background

Both CP and AP are oxidatively bioactivated in vivo by P450s. The aim of this work was to mimic the metabolic process by electrochemical oxidation ex situ. The bioactive intermediate from CP is an electrophilic PM. Its formation involves an initial oxidation to 4-hydroxy-CP catalyzed by CYP2B6. The hydroxy derivative undergoes a spontaneous reverse Michael reaction, yielding the PM and acrolein.[18] Oxidation at other sites on CP leads only to detoxication and excretion. Paci et al. oxidized CP electrochemically to

4-methoxycyclophosphamide in methanol but did not study electrolysis in water.[24]

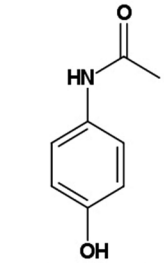


Cyclophosphamide (CP)

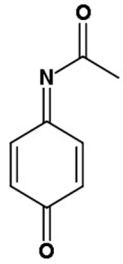


Phosphoramide Mustard (PM)

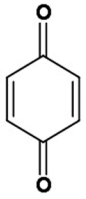
The initial oxidation product of AP is *N*-acetyl-*p*-quinone-imine (NAPQI), whose formation is catalyzed by several P450s in the liver,[20,25] causing hepatic toxicity through adduct formation with biological macromolecules during acute intoxication. NAPQI hydrolyzes to BQ, which also exhibits toxicity through adduct formation [7]. BQ adducts are not prominent metabolic products of AP because NAPQI reacts with cellular nucleophiles competitively with hydrolysis to BQ.[19,20] Other relevant examples include the hydroxylation of diclofenac,[26] the N-dealkylation of imipramine and lidocaine,[27,28] and the phosphothionate oxidation of parathion.[29]



Acetaminophen (AP)



N-acetyl-*p*-quinone-imine (NAPQI)



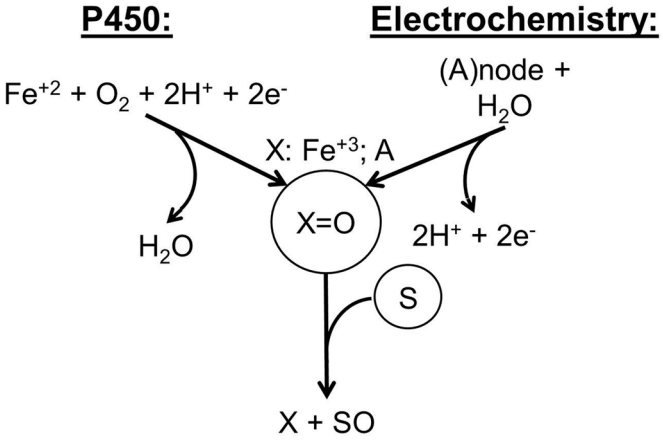
1,4-Benzoquinone (BQ)

The mechanism of prodrug activation by P450s involves binding one atom of molecular oxygen to an iron atom in the catalytic site of the enzyme followed by transfer of the iron-bound oxygen atom to the prodrug. Certain anode materials ("active" anodes)[30] mimic the mechanism of P450s by first oxidizing water to afford an oxidized anode surface from which an oxygen atom is transferred to the substrate[31–33] (Scheme 1). These materials include graphite and dimensionally stable anodes, the latter of which comprise micrometre layers of noble metal oxides on a base of titanium metal (e.g., Ti/IrO_2–Ta_2O_5 and Ti/RuO_2).

The mouse mammary adenocarcinoma cell line EMT6, which is derived from glandular epithelial cells lining milk ducts, was chosen because it readily forms solid tumors when transplanted into naive mice[34,35] in a manner that parallels the progress of human breast cancer.[36–39] In addition, detailed descriptions of the response of EMT6 cells to chemotherapy, including with CP, are available both in vivo and in vitro,[37–39] and the fact that EMT6 was initially derived from a commonly used, commercially available mouse strain (BALB/c)[36] makes it a promising model for future studies for testing EAC in vivo.

We assessed cell viability by measuring the cellular production of NAD(P)H. NA(D)PH reduces a colorimetric dye (MTS) to a soluble formazan, which is actively exported from the cell.[40,41] The electron coupling reagent phenazine methosulfate links the production of NAD(P)H to the reduction of the dye; hence the assay depends indirectly on mitochondrial function of viable cells.[40] A 24 h incubation time was used in toxicity experiments to ensure cells were treated under a subconfluent state, making them more likely to respond to DNA-damaging agents such as CP that target dividing cells.

Scheme 1. Conceptual parallel between the oxygen transfer reactions cytochrome P450 catalytic centers and electrochemical oxidation. X, Fe^{+3}; A, anode surface; S, substrate.



Results and discussion

For optimization of the analytical conditions, EMT6 cells were plated at densities of 2500, 5000, 10 000, and 15 000 cells per 100 μL for 18 h and then treated in the absence or presence of 2 ng mL^{-1} transforming growth factor beta (TFG-β), which inhibits EMT6 growth (A. Viloria-Petit, unpublished observations). In untreated controls the absorbance at 490 nm increased with increasing cell density and incubation time. Cells treated with TGF-β (positive controls) showed ~30% reduction in viability (Fig. 2). The best resolution between TGF-β treated versus control cells was found for a plated cell density of 5000 cells per 100 μL and an MTS reagent incubation time of 2 h.

Control experiments demonstrated a lack of chemical reactivity of MTS towards AP, BQ, and CP, the previous two of which are known to be capable of redox cycling.[19,20] Under cell-free conditions, BQ produced a significant increase in absorbance compared with controls (data not shown), but when the experiment was repeated with milliQ water instead of growth medium, this effect was not seen. This suggested that BQ had oxidized components of the growth medium (e.g., amino acids), thereby being reduced to hydroquinone, which in turn reduced the MTS reagent. Additional control solutions were implemented during the toxicity testing of BQ.

Dose–response curves

Neither CP nor AP decreased cell viability relative to controls when incubated with subconfluent cells for 24 h (at 10–500 μmol L^{-1}; data not shown). Visual inspection revealed that the treated cells remained spindle-shaped and subconfluent. The corresponding experiment with BQ produced a concentration-dependent decrease in cell viability (Fig. 3); under microscopic examination, the cells had a speckled appearance and their morphology was retracted from their neighbors, suggesting an apoptotic mechanism of cell death. Probit analysis of BQ data gave IC_{50} and IC_{99} values of 26 and 138 μmol L^{-1}, respectively (Fig. 3, insert). A quantitative experiment was not possible for CP because authentic oxidation products were unavailable.

Electrochemical oxidation of CP and AP

Prior to electrolysis of the prodrugs themselves, control experiments showed that electrolysis in cell culture medium could not be used because polymeric deposits formed on the anode. Buffer solutions containing chloride (saline) were not used because the oxidation of chloride ions produces hypochlorite, a potent oxidizing and chlorinating agent.[42] Phosphate buffers were unsatisfactory due to chemical reaction with both AP and BQ (data not shown). Sodium sulfate (25 mmol L^{-1}) was used as the supporting

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Fig. 2. Response of MTS assay on plated EMT6 cell density and growth inhibition by TGF-β. (A) Control; (B) 2 ng μL^{-1} TGF-β, n = 3. Absorbance read at 490 nm.

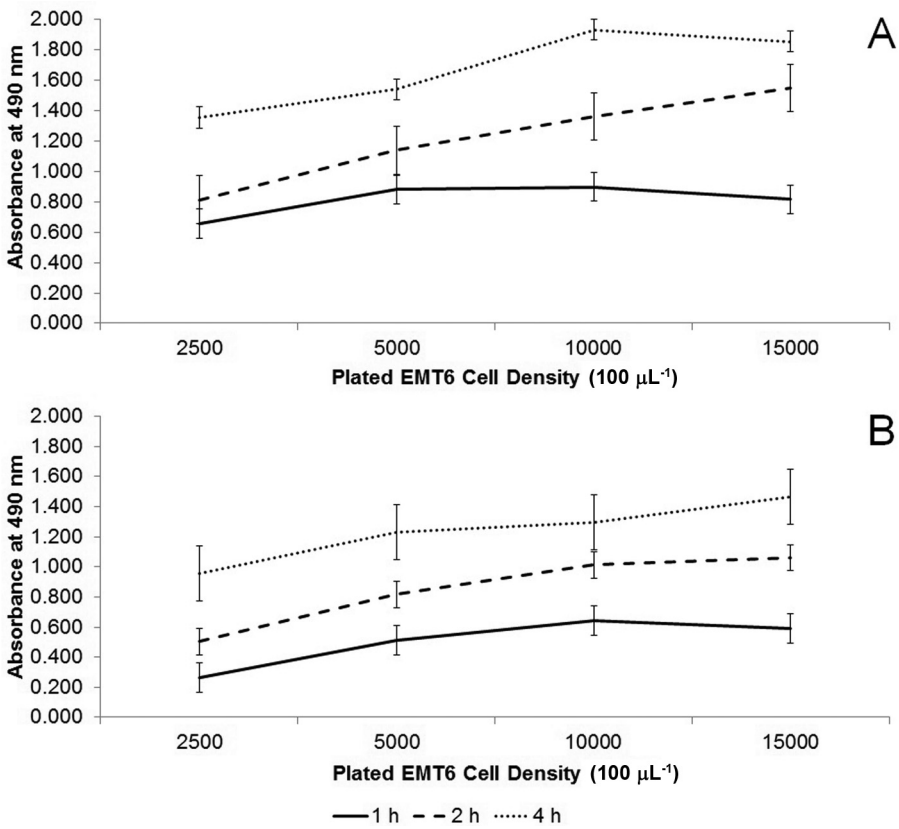
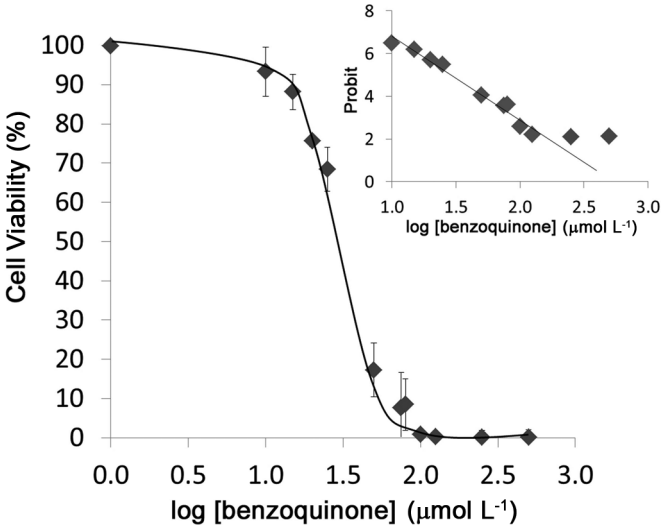


Fig. 3. Viability of 1,4-benzoquinone-treated EMT6 cells; insert: probit transformation. Data points at 250 and 500 μ mol L^{-1} were not included due to the lack of resolution of the MTS assay.



electrolyte in all experiments, as it was inert towards prodrugs and electrolysis products.

Linear sweep voltammetry of CP at either Ti/RuO_2 or graphite microelectrodes gave no oxidation current in the range of water stability. Previously, Paci et al.[24] obtained a voltammetric current for CP in acetonitrile on glassy carbon at +2.0 V (versus SCE), but this potential is well beyond the range of water stability at either graphite or Ti/RuO_2. Amperostatic electrolyses of 0.5 mmol L^{-1} CP solutions (50 mL) were carried out at 5 and 20 mA, with samples taken every 30 min and derivatization with DDTC because the oxidation products themselves have no chromophore. Concurrent oxidation of water (shown by the appearance of oxygen bubbles) was observed at Ti/RuO_2 at both 5 and 20 mA but not at graphite. The chromatograms of the electrolyzed solutions consistently showed a series of inseparable peaks whose identities were presumed to be mono- and di-DDTC–PM adducts of CP oxidation products (Fig. 4).

AP gave a well-defined voltammetric oxidation current at +0.9 V (versus SCE) at graphite as reported previously;[43] this peak was subsumed by water oxidation at Ti/RuO_2 (data not shown). AP was electrolyzed under both potentiostatic and amperostatic conditions using either graphite or Ti/RuO_2 anodes. During potentiostatic electrolyses at a fixed potential of +1.0 V (versus SCE), the rates of loss of starting AP concentration (1.0 mmol L^{-1}) was the same at both graphite and Ti/RuO_2 anodes, consistent with mass-transport limited kinetics. BQ, the only identifiable product, was formed in ~50% yield (somewhat greater at graphite than at Ti/RuO_2), but these yields were unsatisfactory for toxicity studies because of the 1:3 dilution of electrolyzate in the experimental design (see Toxicity assays section above); a concentration of 414 μmol L^{-1} BQ in the electrolyzate is needed to achieve the IC_{99} for EMT6 cells.

Amperostatically, the initial rate of loss of 1.0 mmol L^{-1} AP was independent of the current over the range of 20–80 mA; the rates of oxidation were not greatly different from those of the potentiostatic experiments. At graphite, the reaction tended to slow down, possibly due to oxidation of the anode surface at potentials greater than ~1.5 V (versus SCE).[43] Electrolyses of 5 mmol L^{-1} AP yielded a significantly higher concentration of BQ, especially at graphite, with optimum results obtained at a graphite anode and 20 mA current (Table 1).

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Fig. 4. Reverse-phase HPLC chromatograms of 0.5 mmol L^{-1} cyclophosphamide electrolyzed at 5 mA with a Ti/RuO_2 anode. (A) t = 0 min; (B) t = 30 min; (C) t = 60 min; (D) t = 90 min.

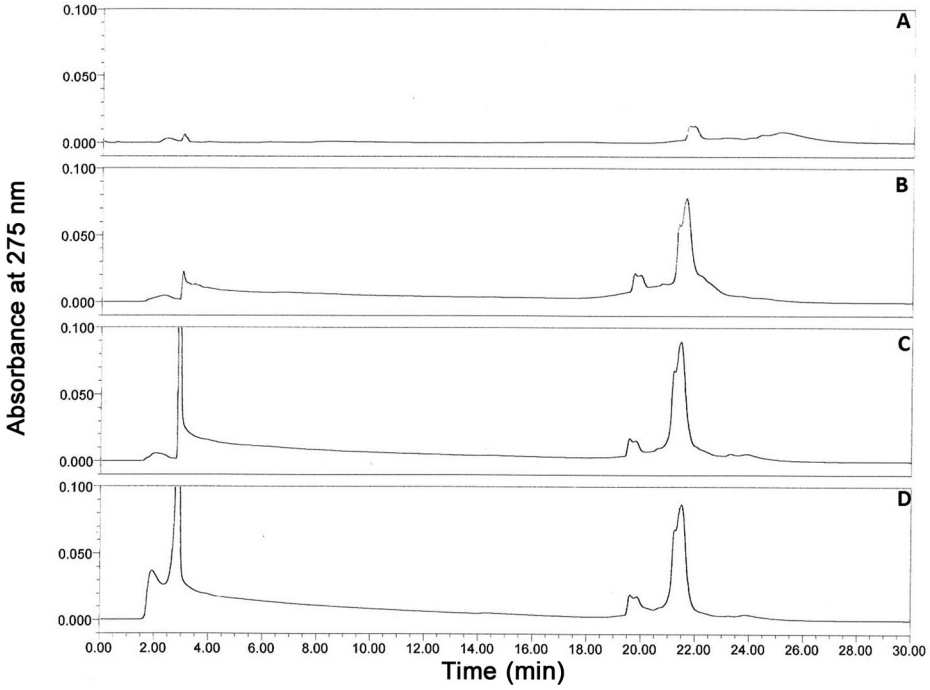

Table 1. Current efficiency and benzoquinone (BQ) yield upon amperostatic electrolysis of acetaminophen (AP).

Anode	AP (mmol L^{-1})	Current (mA)	Time (min)[a]	$[BQ]_{max}$ (μmol L^{-1})[b]	$[BQ]_{max}$ yield (%)	BQ current efficiency (%)[c]
Graphite	1	20	57	430	43.0	43.0
		40	>60	349	34.9	34.9
		80	>60	323	32.3	32.3
	5	20	11.5	1630	32.6	32.6
		40	13.5	1550	31.0	31.0
		80	13.5	1270	25.4	25.4
RuO_2	1	20	>60	265	26.5	26.5
		40	>60	364	36.4	36.4
		80	56	431	43.1	43.1
	5	20	>60	330	6.6	6.6
		40	>60	303	6.1	6.1
		80	>60	330	6.6	6.6

[a]Electrolysis time required to reach IC_{99} (414 μmol L^{-1}).
[b]Highest[BQ] produced during 60 min of electrolysis.
[c][BQ] produced as a stoichimetric ratio to [AP] lost after 60 min of electrolysis.

Cytotoxicity of electrolyzed solutions in a batch cell

No significant toxicity relative to untreated cells was seen in control electrolyses of sodium sulfate (25 mmol L^{-1}) alone in milliQ water. Although the pH of electrolyzed control solutions ranged from 3.0 to 3.5, the culture medium was sufficiently buffered that no toxicity was observed due to the effects of pH alone. Cytotoxicity was not observed at 1 mmol L^{-1} starting concentrations of both CP and AP, but both prodrugs were lethal to greater than 97% of cells after 15 min of electrolysis time at 5 mmol L^{-1} starting concentrations (Fig. 5). Visually, the latter cells were round in shape, speckled in complexion, and retracted from other adjacent cells in response to both compounds. The higher concentrations of prodrug were needed to correct for the 1:3 dilution of the electrolyzate.

Cytotoxicity of electrolyzed solutions in the flow cell

Table 2 compares the cell parameters of the batch and flow cells. Electrolyses using the flow cell produced effluent pH values ranging from 3.0 to 3.5, as in the batch cell experiments. The smaller anodes in the flow cell gave higher current densities which resulted in the gradual degradation of the graphite anode,[43] with particles flaking off and clogging the bore of the needle. However, the cell could be run continuously for >6 h by limiting the current to 5 mA, and under these conditions the current densities used in both batch and flow systems were similar (Table 2). The Ti/RuO_2 anode could not be used in the flow cell because excessive production of O_2 gas obstructed the flow of the anolyte, and so no attempt was made to match the current densities in that system.

Concentrations of 1 and 5 mmol L^{-1} AP or CP were electrolyzed at 5 mA in 25 mmol L^{-1} sodium sulfate using the graphite anode. One millilitre effluent samples were collected (after 1 h of electrolysis time to ensure that a steady-state concentration of effluent had been reached). The flow rate, based on weighing a sample collected over a defined time, was 230 μL min^{-1}, corresponding to

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Fig. 5. EMT6 cell viability after treatment with electrolyzed solutions of (A) acetaminophen and (B) cyclophosphamide in batch cell; initial concentrations of 1 and 5 mmol L^{-1} were used at a graphite anode, with a current of 20 mA. Percent viability corrected relative to untreated cells (50.0 μL of milliQ water). *$p > 0.05$ (Student t test).

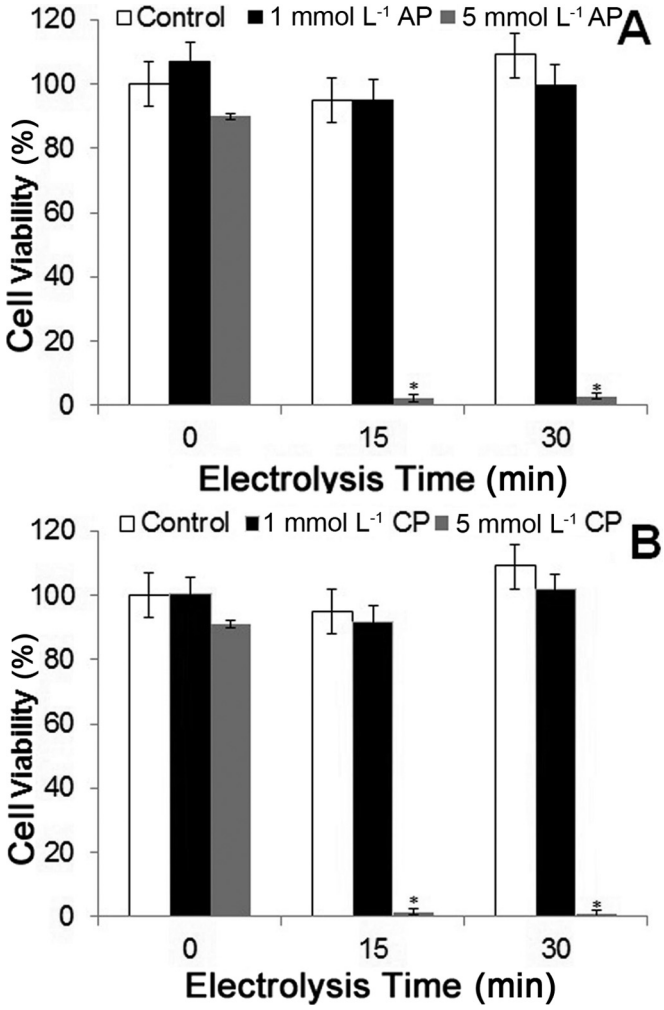
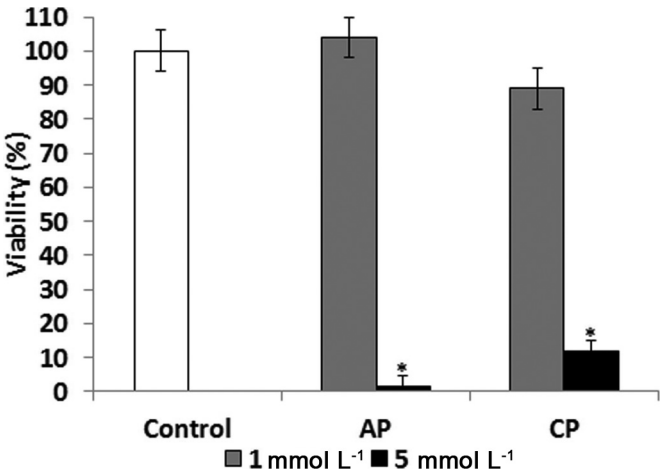


Table 2. Electrode surface areas and current densities used for flow and batch systems.

Anode	Cell	Submerged surface area (cm^2)	Current (mA)	Current density (mA cm^{-2})
Graphite	Batch	20.0	20.0	1.0
	Flow	4.7	5.0	1.1
Ti/RuO_2	Batch	5.3	20.0	3.8
	Flow	3.2	5.0	1.6

a residence time of 4.35 min. Fifty microlitres of effluent was dripped into a culture of subconfluent cells to determine the flow rate of the anolyte. The results with the flow cell corresponded closely to those in the batch electrolyses. No toxicity was observed in control experiments with supporting electrolyte alone; effluents from 1 mmol L^{-1} either prodrug did not significantly affect cell viability (Fig. 6), but the effluent from 5 mmol L^{-1} AP caused greater than 97% reduction of cell viability. With 5 mmol L^{-1} CP, the MTS assay indicated that 12% of cells remained viable after the 24 h incubation, and visual inspection confirmed small, localized colonies of spindle-shaped, adherent EMT6 cells. This may indicate sub-

Fig. 6. EMT6 cell viability after treatment with electrolyzed solutions of acetaminophen and cyclophosphamide in flow cell; initial concentrations of 1 and 5 mmol L^{-1} were used at a graphite anode, with a current of 5 mA. Percent viability corrected relative to untreated cells (50.0 μL of milliQ water). *$p > 0.05$ (Student t test).



populations of treatment-resistant cells: a precedent is the variable response of cultured EMT6 cells towards cyclophosphamide, which was previously suspected to be due to a lack of necessary P450 enzymes in the EMT6 cell line.[44]

Conclusions

We envisage EAC as one member in a suite of localized cancer chemotherapies, all of which focal or "keyhole" applications depend on accurate medical imaging techniques such as magnetic resonance imaging, ultrasound, and positron emission tomography, which are routinely used in the clinic to construct detailed 3D images of tumors in situ.[9–11]

The aim of this work, to provide proof-of-concept for an electrochemical system capable of producing cytotoxic concentrations of toxicant ex situ from nontoxic precursors, was achieved in the electrochemical oxidation of both AP and CP. Their electrolyzed solutions were cytotoxic against EMT6 cells at the IC_{99} level, using both graphite and Ti/RuO_2 anodes, in a manner that mimicked monooxygenase activity. Graphite anodes gave a higher yield of oxidized product from AP than Ti/RuO_2, and the greater potential range of water stability at graphite caused less oxygen gas formation, which was especially important in the performance of the flow cell. For AP, it was possible to correlate the yield of benzoquinone (by HPLC) with the probit curve obtained with the authentic toxicant. The results with AP and BQ confirmed the viability of using "non-DNA-alkylating" toxicants against cancer cells. Although NAPQI is responsible for AP toxicity in vivo, in this work, it was so readily hydrolyzed that it served only as an intermediate for BQ formation. Therefore, the lifetimes of very short-lived, highly reactive toxicants must be considered in future EAC studies.

In summary, we have shown that ex situ electrochemical activation of prodrugs is a novel and potentially useful technology for causing toxicity in tumor cells, many of which lack the P450s to bioactivate prodrugs efficiently. Ex situ oxidation combined with local administration also allows reevaluation of toxicants that have been neglected due to their systemic toxicity.

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